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EXHIBIT 4

RESEARCH ARTICLE

Immunization strategy against cervical cancer involving an alphavirus vector expressing high levels of a stable fusion protein of human papillomavirus 16 E6 and E7

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We are developing immunization strategies against cervical carcinoma and premalignant disease, based on the use of recombinant Semliki Forest virus (SFV) encoding the oncoproteins E6 and E7 from high-risk human papilloma viruses (HPV). Thus far, protein-based, as well as genetic immunization studies have demonstrated low to moderate cellular immune responses against E6 and E7. To improve these responses, we modified the structure and expression level of the E6 and E7 proteins produced by the SFV vector. Specifically, a construct was generated encoding a fusion protein of E6 and E7, while furthermore a translational

enhancer was included (enhE6,7). Infection of cells with recombinant SFV-enhE6,7 resulted in the production of large amounts of the E6,7 fusion protein. The fusion protein was more stable than either one of the separate proteins. Immunization of mice with SFV-enhE6,7 resulted in strong, long-lasting HPV-specific cytotoxic T lymphocyte responses. Tumor challenge experiments in mice demonstrated that immunization with SFV-enhE6,7 resulted in prevention of tumor outgrowth and subsequent protection against tumor re-challenge.

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Introduction

Infection of cervical epithelial cells by the high-risk human papillomavirus (HPV) types 16 or 18 is closely associated with the development of cervical carcinoma. The transforming potential of these viruses depends on the sustained expression of the early viral genes E6 and E7. Since E6/E7 expression is selectively maintained in (pre)malignant cervical lesions, these proteins are attractive candidates for immunotherapeutic strategies.¹ Specific cell-mediated immune responses are believed to be critical for the elimination of HPV-infected cells or HPV-transformed tumor cells. This assumption is based on the observations that in the majority of individuals HPV-induced lesions regress spontaneously, and that immunodeficient patients, like immunosuppressed transplant recipients or AIDS patients, develop significantly more HPV-related lesions in skin and anogenital tissue than immunocompetent individuals.^{2,3} HPV E6 and E7 proteins, constitutively expressed in HPV-transformed cells, are considered to be tumor-specific antigens that can act as targets for cytotoxic T lymphocyte (CTL)-mediated tumor cell killing and stimulation of tumor-specific CTL activity.^{4–7}

The induction of class I MHC-restricted CTL activity is optimally achieved by synthesis of viral antigens within antigen presenting cells (APCs), for example through

immunization with live attenuated virus. However, apart from its potential risk, this is at present not an option for immunization against HPV infection since the virus cannot be successfully propagated to yield virus particles. Several approaches have been described for the induction of HPV-specific CTL responses such as immunization with recombinant viruses, HPV-specific proteins or peptides, E7 linked to heat shock proteins or intracellular sorting molecules, peptides/proteins loaded on dendritic cells, DNA- or RNA-transfected dendritic cells (DCs) or cell lines.^{8–13}

We are exploiting the Semliki Forest virus (SFV) expression system.^{5,14–17} This vector represents an ideal system for the induction of cellular immune responses against the oncoproteins HPV E6 and E7 since, apart from its high efficiency and biosafety, SFV RNA does not integrate and SFV infection is cytolytic by apoptosis. Thus, it is very unlikely that the genetic information for E6 and E7 will persist for prolonged periods of time within the organism.

We recently demonstrated that immunization of mice with recombinant SFV particles encoding HPV16 E6 and E7 resulted in a potent HPV16-specific CTL and anti-tumor response.⁵ However, we were unable to induce full tumor protection. Anti-HPV responses are very low or absent in cervical carcinoma patients, which is believed to be due to immunological tolerance or ignorance.^{18,19} Therefore, effective immunization strategies should induce a very potent immune response to overrule this effect. In the HPV16 genome, the E6 and E7 genes are present in tandem, with a stop codon behind the E6 gene. We have now generated a construct in which the stop

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codon between E6 and E7 was removed and 1 bp was inserted between the genes of E6 and E7 resulting in a construct that codes a stable fusion protein of E6 and E7. Since upon infection with SFV the infected cells die through apoptosis²⁰⁻²³ it is to be expected that the antigen is presented in a process of cross-priming. Therefore a protein should not be completely degraded before the entire process of the infection of cells, apoptosis induction and uptake and processing by an APC has been completed. Since the protein encoded by the novel construct is much more stable than the original E6 and E7 proteins, we hypothesize that immunization with the novel construct might result in an enhanced immune response. In addition, a translational enhancer was included to induce a high production of the fusion protein. In the present study, the characteristics of the fusion protein produced and the efficacy of recombinant SFV particles encoding this protein both with respect to CTL induction and anti-tumor response in a murine HPV-tumor model are studied.

Results

Cloning of HPV16 E6 and E7 in pSFV3

The HPV 16 E6 and E7 genes were obtained by PCR using the plasmid pRSV-HPV16E6E7 as a template. This plasmid was kindly provided by Dr J Ter Schegget (University of Leiden, The Netherlands). In the plasmid, the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. In a previous study we described the construction of the pSFV3-E6E7 plasmid encoding E6 and E7 as separate proteins.⁵ In this plasmid, as in pRSV-HPV16E6E7, the HPV16 E6 and E7 genes are present in tandem with a stop codon after the E6 gene. In the present study, we generated a novel recombinant SFV plasmid. This plasmid encodes a fusion protein of E6 and E7 by inserting 1 bp between E6 and E7 and by changing the stop codon of E6. In addition, a translational enhancer is included in the construct. This new plasmid is named pSFV3-enhE6,7. As a control, we also generated a construct in which the translational enhancer was placed in front of the original E6E7 construct, pSFV3-enhE6E7.

Western blot analysis

To verify that SFV-enhE6,7 induces expression of a recombinant fusion protein of E6 and E7, lysates of cells infected with SFV-enhE6,7 were analyzed by Western blotting. In addition, lysates of cells infected with the original construct, SFV-E6E7 or with the construct in which the translational enhancer was cloned in front of the original E6E7 construct, ie SFV-enhE6E7, were analyzed.

In Figure 1, Western blots probed with anti-HPV16 E6 or anti-HPV16 E7 are shown. The conditions were optimized for detection of the abundant expression of E6 and E7 by the vectors containing the translational enhancer. In lysates from cells infected with SFV-enhE6,7 three prominent bands were revealed with both the anti-E6 and the anti-E7 antibody (Figure 1, lanes 3 and 6). By contrast, no or very little E6 and E7 could be demonstrated upon infection with SFV-E6E7 (Figure 1, lanes 1 and 4). Likewise, in a previous study, expression of E6 and E7 upon infection with SFV-E6E7 could be demon-

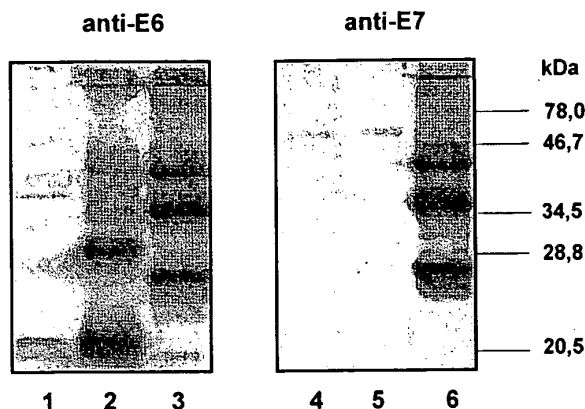


Figure 1 Western blot analysis of recSFV transfected BHK cell extracts. BHK cells were infected with SFV-enhE6,7, SFV-E6E7 or SFV-enhE6E7 particles. After overnight incubation, the cellular proteins were extracted and analyzed by SDS-PAGE and immunoblotting. E6 was detected using a polyclonal rabbit-anti-HPV16 E6 antibody (lanes 1–3); E7 was detected using a monoclonal mouse-anti-HPV16 E7 antibody (lanes 4–6). Lanes 1 and 4, BHK21 cells infected with SFV-E6E7 particles; lanes 2 and 5, BHK21 cells infected with SFV-enhE6E7 particles; lane 3 and 6, BHK21 cells infected with SFV-enhE6,7 particles. Given is the kDa of marker proteins.

strated only when using larger samples and a prolonged staining time.⁵ In cells infected with SFV-enhE6E7 two prominent bands stained with the anti-E6 but not with the anti-E7 antibody (Figure 1, lanes 2 and 5, respectively). This suggests that, as expected, the translational enhancer only increases E6 synthesis while, due to the stop codon in between E6 and E7, E7 is not translated in an enhanced fashion.

The three major bands observed in the SFV-enhE6,7 lysates had apparent electrophoretic mobilities of approximately 26 kDa, 36 kDa and 44 kDa, respectively. The 26 kDa band presumably represents the fusion protein of E6 and E7 (17 kDa and 11 kDa, respectively). Since the 36 and 44 kDa bands stain positive with the anti-E6 antibody, as well as with the anti-E7 antibody, these bands presumably represent high order complexes of the E6,7 fusion protein. In this regard it should be noted that, as we and others have demonstrated, that the electrophoretic mobility of the E7 protein does not correspond to the calculated molecular weight (MW).^{5,24} Similarly, the apparent electrophoretic mobility of the bands may not reflect the actual MWs of the fusion proteins. The two major E6 bands observed in lysates from cells infected with SFV-enhE6E7 had apparent electrophoretic mobilities of approximately 21 kDa and 34 kDa. The 21 kDa band is slightly higher than the calculated MW of E6, ie 17 kDa. The 34 kDa band might represent a dimeric complex of E6.

Analysis of E6 and E7 expression by pulse labeling

Production and stability of the fusion protein E6,7 by BHK cells infected with SFV particles was analyzed by pulse-chase-labeling of the cells with ³⁵S-methionine/cysteine. As shown in Figure 2 (lane 6), infection of BHK cells with SFV-enhE6,7 particles and radiolabeling for 1 h, resulted in three prominently labeled bands of the E6,7 fusion protein. These bands correspond to the bands revealed on the Western blots. The

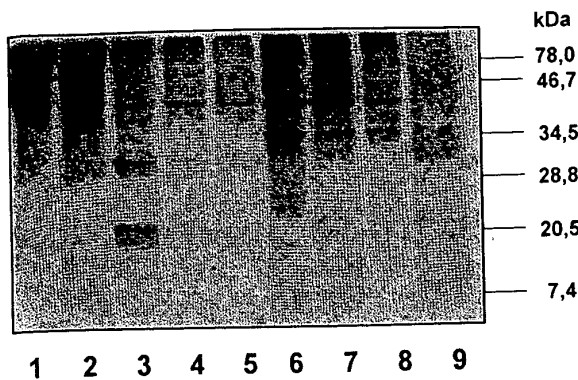


Figure 2 Analysis of E6 and E7 expression by pulse labeling. BHK cells were infected with SFV-enhE6,7, SFV-E6E7 or SFV-enhE6E7 particles. After 6 h, the cells were cultured for an additional 30 min with methionine- and cysteine-free medium followed by a 1-h labelling-period with ^{35}S -methionine/cysteine. After 1 h, the cells were washed and harvested or cultured for an additional 6, 16 or 40 h before harvesting. Cell lysates were analyzed by autoradiography. Lane 1, BHK21 cells not infected, analyzed after a 6-h chase; lane 2, BHK21 cells infected with SFV-E6E7, analyzed after a 6-h chase; lanes 3–5, BHK cells infected with SFV-enhE6E7, analyzed directly or after a 6-h or 16-h chase, respectively; lanes 6–9, BHK21 cells infected with SFV-enhE6,7, analyzed directly or after a 6-h, 16-h or 40-h chase, respectively.

44-kDa band runs slightly lower than the upper band in lanes 1–5. The bands of 36 and 44 kDa are still present after a 6- and 16-h chase period (Figure 2, lanes 7 and 8, respectively). Even after a 40-h chase period both bands remain visible (Figure 2, lane 9). By contrast, under the conditions of the experiment, the E6 and E7 proteins produced upon infection with SFV-E6E7, either following a 6-h chase period (Figure 2, lane 2) or directly upon labeling were undetectable (not shown). Previously, we demonstrated that prolonged exposure times of the film are needed to visualize these proteins.

Autoradiography of lysates from cells infected with SFV-enhE6E7 directly after labeling (Figure 2, lane 3) revealed the same bands as those observed by Western blot analysis, i.e. a 21- and a 34-kDa band. However, in contrast to the enhanced fusion protein, within a 6-h chase period most of the E6 protein was degraded. After 16 h the protein was degraded almost completely.

HPV-specific CTLs induced by immunization of mice with SFV-enhE6,7

Mice were immunized s.c. and boosted twice (s.c. and i.p.) with 10^5 or 10^6 purified SFV-enhE6,7 or with SFV-E6E7, SFV-enhE6E7, SFV-LacZ particles or buffer. CTL activity was determined 1 week after the last booster immunization. After 7 days (Figure 3a) and 14 days (Figure 3b) of *in vitro* restimulation, the resulting effector cells were tested for their cytolytic activity against 13-2 target cells and C3 target cells. The C3 cell line contains the complete HPV16 genome. The 13-2 cell expresses an HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF) (see Materials and methods). As shown in Figure 3, spleen cells isolated from mice immunized with 10^6 but also with as few as 10^5 SFV-enhE6,7 particles already displayed a high level of cytotoxicity against 13-2 tumor cells in the short-term restimulation protocol (i.e. 7 days; Figure 3a). Similar levels of cytotoxicity were induced against C3 target cells (not shown). By contrast, upon immunization with 10^6

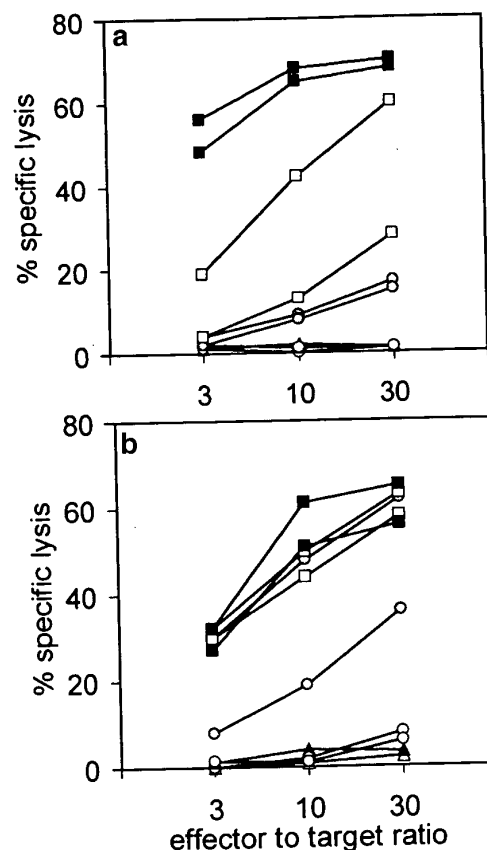


Figure 3 CTL activity induced upon immunization with SFV-enhE6,7 particles versus SFV-E6E7 particles, as determined after a 7- and 14-day *in vitro* restimulation. Mice were immunized s.c. and boosted twice (s.c. and i.p.) with purified 10^6 SFV-enhE6,7 ($n = 2$, closed squares), 10^5 SFV-enhE6,7 ($n = 2$, open squares), 10^6 SFV-E6E7 ($n = 2$, gray circles), 10^5 SFV-E6E7 particles ($n = 2$, open circles) or with 10^6 SFV-LacZ ($n = 2$, closed triangles) or PBS ($n = 2$, open triangles) as controls. CTL activity was determined 1 week after the last booster immunization. After 7 days (a) and 14 days (b) *in vitro* restimulation the resulting effector cells were tested for cytotoxic activity against 13-2 target cells in triplicate well assay. The levels of cytotoxicity at different effector to target ratios are shown.

SFV-E6E7, significant levels of CTL activity could be determined only after long-term restimulation (Figure 3b), short-term restimulation resulting in a very low level of CTL activity. Upon immunization with 10^5 SFV-E6E7, no CTL activity was detectable.

Immunization with SFV-enhE6E7 (particles encoding an enhanced production of E6 and a normal production of E7) did not induce detectable levels of CTL activity in the short-term CTL assay against 13-2 cells nor against C3 cells (not shown).

Next, the level and maintenance of CTL activity induced upon administration of higher dosages of SFV-enhE6,7 particles were determined. Mice were immunized with 1 , 2.5 or 5×10^6 SFV particles and CTL activity was determined 18 days and 8 weeks after the last booster immunization, using the short-term restimulation protocol. As shown in Figure 4, the level of CTL activity induced with 10^6 SFV-enhE6,7 is presumably the maximal level of lysis that can be reached and detected in the bulk CTL ^{51}Cr -release assay as immunization with 2.5

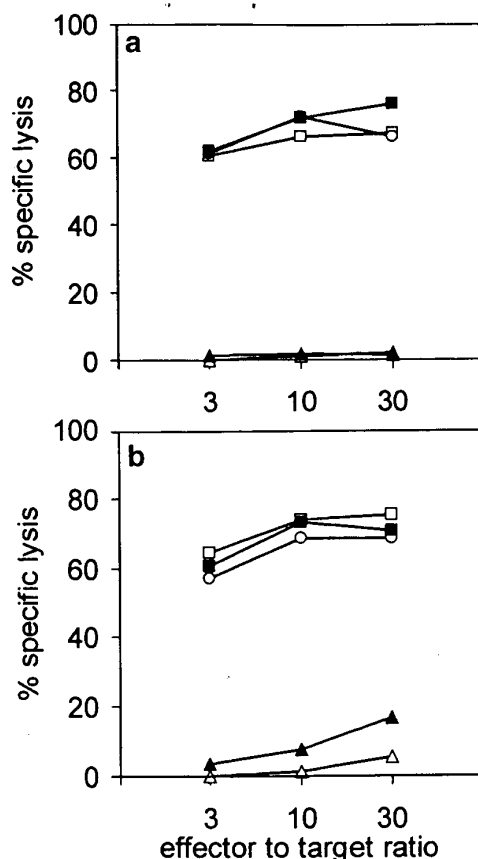


Figure 4 CTL activity induced upon immunization with an increasing dose of SFV-enhE6,7 particles. Mice were immunized s.c. and boosted twice (s.c. and i.p.) with 10^6 SFV-enhE6,7 (open squares), 2.5×10^6 SFV-enhE6,7 (open circles) or 5×10^6 SFV-enhE6,7 particles (closed squares) or with 5×10^6 SFV-LacZ particles (closed triangles) or PBS (open triangles) as controls. CTL activity was determined 18 days (a) or 8 weeks (b) after the last booster immunization. After 7 days *in vitro* restimulation, the resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay. The levels of cytotoxicity at different effector to target ratios are shown.

and 5×10^6 SFV-enhE6,7 did not increase the percentage of specific lysis. Importantly, 8 weeks after the last booster immunization, levels of cytotoxicity were similar to those reached 18 days after the last booster (Figure 4b and a, respectively).

Effect of booster immunizations and routes of immunization on SFV-enhE6,7 induced CTL activity

In previous immunization protocols mice were always immunized three times (ie one primary immunization followed by two booster immunizations, s.c. and i.p.). In order to determine the number of immunizations needed to induce a long-term response, we immunized mice once or twice and determined the level of CTL activity at 10 days, 1 month or 3 months after the last immunization.

Figure 5a shows that a single immunization of 2.5×10^6 SFV-enhE6,7 particles induces a significant level of cytotoxicity at 10 days after immunization (squares). This level gradually decreases over the next 3 months (1 month, circles; 3 months, triangles). However, a single boost suffices to induce a significant CTL response up

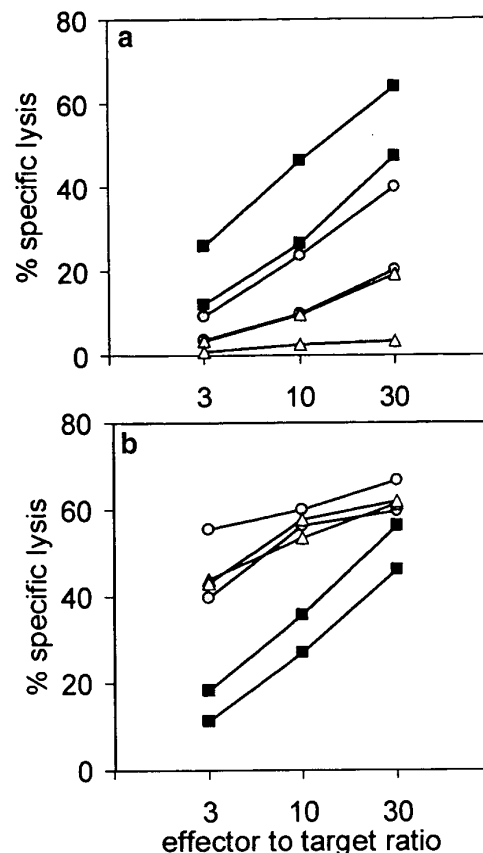


Figure 5 Induction of long-term CTL activity requires a single booster immunization. Mice received a single s.c. injection of 2.5×10^6 SFV-enhE6,7 particles (a) or two s.c. injections of 2.5×10^6 SFV-enhE6,7 particles (b). CTL activity was determined 10 days (closed squares), 1 month (gray circles) or 3 months (open triangles) after the (last) injection of particles. After 7 days *in vitro* restimulation the resulting effector cells were tested for cytotoxicity against 13-2 target cells in triplicate well assay. The levels of cytotoxicity at different effector to target ratios are shown.

to 3 months after the booster immunization (Figure 5b, triangles) which was as high as the response after 1 month (Figure 5b, circles).

Next, the effect of s.c. immunization was compared with i.p. immunization. Mice were immunized s.c. or i.p. with 1×10^6 SFV-enhE6,7 particles. In the bulk CTL assay, the levels of specific lysis by spleen cells isolated from the s.c. immunized mice are comparable to those of mice immunized i.p. (Figure 6a and b).

Determination of CTL precursor frequency by IFN- γ Elispot

For the bulk CTL assay, spleen cells are restimulated *in vitro* for several days resulting in the proliferation of CTL precursors. Therefore, this assay cannot quantify the actual frequency of CTL precursors induced *in vivo*. To evaluate this number of CTL precursors, HPV16 IFN- γ Elispot assays were performed.

As demonstrated in Table 1, the number of CTL precursors 18 days after injection of 1 , 2.5 and 5×10^6 SFV-enhE6,7 particles, as determined by an IFN- γ Elispot assay, was within the range of one in 1780 to one in 6600 total spleen cells. Since approximately 8% of the C57Bl/6

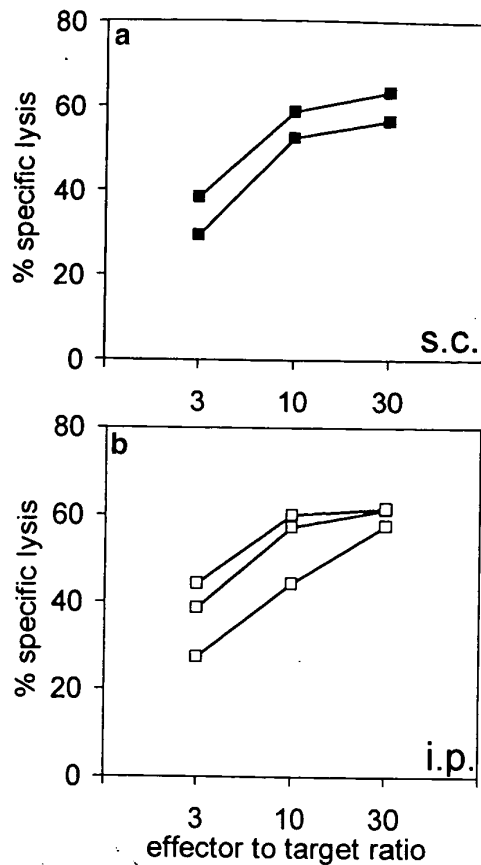


Figure 6 CTL induction upon subcutaneous versus intraperitoneal immunization. Two mice were injected s.c. (a) and three mice were injected i.p. (b) with 10^6 SFV-enhE6,7 particles. CTL activity was determined 10 days after the booster injection against 13-2 target cells. The levels of cytotoxicity at different effector to target ratios are shown.

spleen cells are CD8⁺ T cells (as determined by FACS analysis), this means that, as many as one in 140 to one in 530 CD8⁺ splenic T cells are HPV-specific. Eight weeks after the last booster immunization the level still ranged between one in 430 to one in 1090 CD8⁺ T cells. In mice immunized with 1×10^6 or 5×10^6 SFV-E6E7, the precursor frequency induced was too low to be detected in our Elispot assay, ie fewer than two in 4×10^5 spleen cells plated formed an IFN- γ spot.

Protection against tumor challenge and rechallenge

To examine whether the novel recombinant SFV particles would generate protective immunity against a subsequent tumor challenge, mice were immunized and boosted with SFV-enhE6,7 particles and challenged s.c. with TC-1 cells. The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7.¹¹ Figure 7 shows combined results of two separate immunization studies. Control mice, injected with PBS ($n = 10$) or SFV-LacZ particles ($n = 10$) developed palpable tumors within 2 to 4 weeks after tumor cell inoculation. Immunization with only 10^6 SFV-enhE6,7 particles protected nine out of 10 mice from developing a tumor. Immunization with 5×10^6 particles also resulted in a high degree of protection, four out of five mice remaining tumor-free. In a separate experiment, s.c. immunization with 5×10^6 SFV-E6E7 particles resulted in a partial tumor protection, ie two of five mice did not develop a tumor in 10 weeks.⁵

To determine if long-term protection is induced, mice that did not develop a tumor were rechallenged s.c. with 2×10^4 tumor cells at week 13 or at week 25 after the initial tumor challenge. As shown in Table 2, all mice immunized s.c. with 5×10^6 SFV-enhE6,7 that did not develop a tumor at the initial tumor challenge were protected against the second tumor challenge 13 weeks later. Of the mice immunized with 10^6 SFV-enhE6,7, 60% and 50% did not develop a tumor upon a second tumor challenge at week 13 and week 25, respectively. In a separate experiment we demonstrated that a s.c. growing TC-1 tumor by itself does not induce a detectable CTL response, nor does it enhance the CTL response induced

Table 1 Precursor CTL frequency in SFV-enhE6,7 immunized mice as determined by IFN-gamma Elispot assay

Immunization ^a	Dose	Evaluation time point	pCTL frequency total spleen ^b	PCTL frequency in CD8 ⁺ T cells ^c
SFV-enhE6,7	1×10^6	18 days	1 in 6557	1 in 524
SFV-enhE6,7	2.5×10^6	18 days	1 in 1785	1 in 143
SFV-enhE6,7	5×10^6	18 days	1 in 4081	1 in 326
SFV-enhE6,7	1×10^6	8 weeks	1 in 5381	1 in 430
SFV-enhE6,7	2.5×10^6	8 weeks	1 in 13636	1 in 1090
SFV-enhE6,7	5×10^6	8 weeks	1 in 7692	1 in 615
SFV-E6E7	1×10^6	11 days	<2 in 4×10^5	<1 in 16×10^3
SFV-E6E7	5×10^6	11 days	<2 in 4×10^5	<1 in 16×10^3
SFV-E6E7	1×10^6	18 days	<2 in 4×10^5	<1 in 16×10^3
SFV-LacZ	5×10^6	18 days	0 in 4×10^5	—
PBS		18 days	0 in 4×10^5	—

^aMice were immunized s.c. and boosted twice (s.c. and i.p.) with 1 , 2.5 or 5×10^6 SFV-enhE6,7 or SFV-E6E7 or with 5×10^6 SFV-LacZ or PBS as controls.

^bSpleen cells were isolated 11 days to 8 weeks after the last booster immunization. The frequency of precursor CTLs was determined by INF-gamma Elispot assay upon overnight *in vitro* stimulation with 13-2 cells expressing HPV16-E7 49-57 (MHC class I epitope).

^cCalculated frequency based on a CD8 frequency of 8% of the total spleen as determined by FACS analysis (not shown).

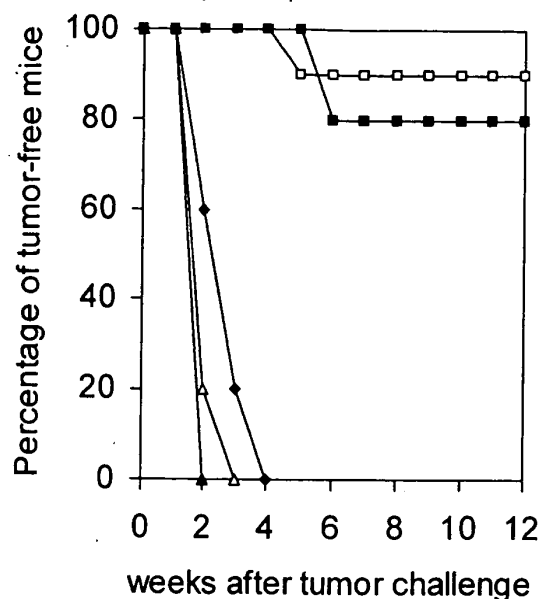


Figure 7 Protection from tumor growth in SFV-enhE6,7 immunized mice. Mice were immunized s.c. and boosted twice (s.c. and i.p.) with 1×10^6 SFV-enhE6,7 particles ($n = 10$; open squares), 5×10^6 SFV-enhE6,7 particles ($n = 5$; closed squares), 10^6 SFV-E6E7 particles ($n = 5$; closed diamonds), 10^6 SFV-LacZ particles ($n = 10$; closed triangles) or PBS ($n = 10$; open triangles). One week after the last booster immunization, 2×10^4 TC-1 cells were inoculated s.c. in the neck. Tumor growth was monitored twice weekly. The percentages of mice with non-palpable tumors in time are shown.

by SFV-enhE6,7. Thus, the rejection of the second tumor challenge is most likely not influenced by the first tumor cell inoculum.

Discussion

Here we demonstrate the potency of a recombinant SFV vector system in the induction of a long-lasting cellular immune response against HPV-transformed tumor cells.

This immune response protects mice from a subsequent challenge and rechallenge with tumor cells.

The novel SFV-enhE6,7 vector described in the present study induces production of large amounts of a relatively stable fusion protein of HPV16 E6 and E7. Pulse-labeling experiments demonstrated that while E6 and E7 separately are degraded almost completely within 6 h after synthesis, the E6,7 fusion protein is degraded much more slowly. Up to 40 h after synthesis the labeled fusion protein can be detected by autoradiography. The CTL response and anti-tumor activity induced by SFV-enhE6,7 particles is much stronger compared with the responses induced with SFV-particles, producing the separate E6 and E7 proteins (SFV-E6E7 and SFV-enhE6E7). Thus, the unique properties of the protein produced by SFV-enhE6,7 give rise to a significantly improved processing and presentation of the antigen to immune effector cells.

Enhancement of CTL induction upon immunization with a vector encoding a more stable protein may seem inconsistent with several excellent studies in which MHC class I presentation has been demonstrated to be potentiated by enhanced degradation of antigen.²⁵⁻²⁹ However, the explanation lies in the presumed pathway through which antigen is presented upon injection of recombinant SFV particles. SFV can infect a wide range of host cells by receptor-mediated endocytosis. Since upon infection with SFV the infected cells die through apoptosis,²⁰⁻²³ it is to be expected that the antigen is presented in a process of cross-priming. Cellular debris from infected cells taken up by an APC will result in processing and presentation by both MHC class I and class II molecules.^{30,31} Thus, the recombinant proteins to be presented should be stable for the time that is required for the entire process of infection of cells through MHC presentation by APCs. Although RNA replication and translation occur within 6 h after infection, killing of the infected cells by apoptosis takes another 24 to 72 h.²⁰ It therefore takes at least 24 h after production before the protein gradually becomes available for APCs to be presented. Thus, the balance between stability and rate of degradation of the protein will determine the efficiency of antigen presentation.

Table 2 Protection from growth of TC-1 tumor cells in SFV-enhE6,7 immunized mice upon tumor challenge and rechallenge

Immunization ^a	Number of tumor-free mice after first tumor challenge/total number of mice ^b	Number of tumor-free mice after second tumor challenge/total number of mice ^c
Exp. 1 Tumor challenge day 0, tumor rechallenge week 25		
SFV-enhE6,7 (1×10^6)	4/5	2/4
SFV-E6E7 (1×10^6)	0/5	—
SFV-LacZ (1×10^6)	0/5	—
PBS	0/5	0/3 ^d
Exp. 2 Tumor challenge day 0, tumor rechallenge week 13		
SFV-enhE6,7 (1×10^6)	5/5	3/5
SFV-enhE6,7 (5×10^6)	4/5	4/4
SFV-LacZ (5×10^6)	0/5	—
PBS	0/5	0/3 ^d

^aMice were immunized s.c. and boosted twice (s.c. and i.p.) as indicated in the table. One week after the last booster immunization, 2×10^4 TC-1 tumor cells were inoculated s.c. in the neck.

^bTumor growth was monitored twice weekly. The numbers of tumor-free mice per total number of mice per group are shown.

^cMice that remained tumor-free were subsequently rechallenged with 2×10^4 TC-1 cells, in the first experiment in week 25, in the second experiment in week 13 after the first tumor challenge.

^dIn the rechallenge experiments, three age-matched control mice were included.

It should be noted that the new construct not only encodes for a more stable protein, it also encodes a translational enhancer. Sjöberg *et al*³² demonstrated that inclusion of this enhancer resulted in an approximately 10-fold increased production of recombinant protein per infected cell. One can therefore argue that the enhanced immune response might solely be ascribed to the larger amount of protein produced. On the other hand, Colmenero *et al*³³ demonstrated that SFV particles encoding P815A antigen resulted in a marginally better protective anti-tumor effect with a construct that contained the enhancer sequence as compared with a construct without enhancer. Thus, the enhanced immune response of SFV-enhE6,7 as compared with SFV-E6E7 is presumably not merely due to the enhanced protein production. Nonetheless, it is likely that the enhanced production of the protein adds to the benefits of the stable fusion protein with respect to efficacy of antigen presentation.

In recent years, a variety of immunization strategies against HPV-transformed tumor cells has been developed and explored. Despite the induction of CTL responses against HPV-transformed tumor cells with these vaccines, the induction of therapeutic anti-tumor responses in tumor-bearing mice has been proven to be more difficult. Recent studies have demonstrated that protective immunity depends on an optimal presentation of tumor antigens to the immune system, not only by MHC class I molecules but also by class II molecules.^{11,34-37} Modification of peptides, proteins and DNA constructs resulting in targeting of antigenic peptides to the MHC class II compartments resulting in CD4⁺ helper responses has been demonstrated to strongly enhance tumor immunity, as well as maintenance of CTL memory.^{38,39} The SFV vector system would appear to be an ideal system for inducing such optimal immunity, since, as described above, SFV infection results in apoptosis of infected cells that will be taken up by specialized antigen presenting cells (APCs). In a process called cross-presentation, these APCs will process peptides for both MHC class I and MHC class II presentation. This results not only in the activation of CD8⁺ T cells, but also the activation of antigen specific CD4⁺ T cells that contribute to the efficient priming of specific CTLs.

For clinical evaluation of SFV-based HPV immunization strategies, biosafety of the vector system, as well as the potential oncogenicity of E6 and E7, are major concerns. However, in the last decade Liljeström and colleagues have generated several SFV vector systems with increasing levels of biosafety.^{14,16,32,40} The most recent vector has a theoretical frequency of wild-type revertants of 10⁻¹⁷.⁴⁰ Several murine and primate studies have demonstrated the efficacy as well as the biosafety of the SFV vector system.^{5,15,17,32,41-43} Thus far the SFV vector system has not been evaluated in humans. With respect to the potential oncogenicity of E6 and E7, it should be noted that cellular transformation requires the constitutive expression of both proteins. The SFV vector system minimizes the risk of constitutive expression of recombinant proteins, since SFV replication leads to death by apoptosis of infected cells and SFV RNA does not integrate in the host genome. Yet, one could consider mutating, deleting or scrambling specific regions of E6 and E7, thereby abolishing the oncogenic properties of the proteins.^{8,44,45}

In conclusion, we have demonstrated that immuni-

zation of mice with a recombinant SFV vector encoding a fusion protein of HPV 16 E6 and E7 results in a potent anti-tumor response capable of preventing tumor outgrowth. Its efficiency and high level of biosafety justifies clinical evaluation of SFV-enhE6,7 particles in cervical cancer patients.

Materials and methods

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (No. CCL-10). The cells were grown in GMEM (Life Technologies, Paisley, UK) containing 5% fetal calf serum (PAA Laboratories, Linz, Austria). C3 cells, 13-2 cells and TC-1 cells were kindly provided by Dr C Melief and Dr R Offringa (Leiden University, The Netherlands). The C3 cell line was derived from C57BL/6 (H-2^b) embryonic cells transfected with a plasmid containing the complete HPV16 genome. The 13-2 cell line was generated from C57BL/6 (H-2^b) embryonic cells transfected with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF) (R Offringa, personal communication). The TC-1 cell line was generated from C57BL/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-ras.¹¹ C3, 13-2 and TC-1 cells were grown in IMDM (Life Technologies) supplemented with 10% fetal calf serum. Both media contained penicillin and streptomycin (Life Technologies; 100 U/ml and 100 µg/ml, respectively).

Mice

Specific pathogen-free female C57BL/6 mice (Harlan CPB, Zeist, The Netherlands) were between 6 and 10 weeks of age at the start of the immunization protocols.

Construction of pSFV3-E6E7, pSFV3-enhE6,7 and pSFV-enhE6E7

pSFV-Helper 2 was kindly provided by Dr P Liljeström (Karolinska Institute, Stockholm, Sweden).¹⁶ pSFV3 was obtained from Life Technologies. The HPV16 E6 and E7 genes were obtained from the plasmid pRSV-HPV16E6E7, which was kindly provided by Dr J Ter Schegget (Amsterdam Medical Center, Amsterdam, The Netherlands).⁴⁶ In this plasmid the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. Amplification of the E6E7 tandem gene was done by PCR. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pGEM7Zf+. After sequence confirmation, the E6E7 fragment was cloned into the unique *Bam*HI site of pSFV3, producing pSFV3-E6E7.

The plasmid pSFV3-enhE6,7 was generated to express high levels of a fusion protein of HPV16 E6 and E7 by including a translational enhancer. The construction is described as follows. Out of the pSFV3-E6E7 the E6 sequence was modified with a *Nco*I site at the 5' end and an *Eco*RI site at the 3' end. The E7 sequence was modified with an *Eco*RI site at the 5' end and a *Bam*HI site at the 3' end by PCR. The 5' end of the capsid gene of SFV coding for the first 34 amino acid residues has been shown to contain a translational enhancer.³² This

enhancer was cloned in a pSFV-helper-S1 construct by Smerdou and Liljestrom.⁴⁰ In addition they inserted the sequence of foot-and-mouth disease virus (FMDV) 2A autoprotease (17 amino acids) in frame between the translational enhancer and p62 (SFV envelope protein) in order to provide cleavage between the proteins. We synthesized the sequence containing the translational enhancer and the FMDV A2 autoprotease from pSFV-helper-S1, and by PCR *Bam*HI and *Nco*I restriction sites were generated at the 5' and 3' end, respectively. The enhFMDV A2 protease-, E6 and E7 fragments were cloned into the *Bam*HI site of pSFV3. In the original plasmid, the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. In pSFV3-enhE6,7 1 bp is inserted between E6 and E7 and the stop codon TAA of E6 is changed into GAA. Thus, in pSFV3-enhE6,7 the sequence encoding E6 and E7 is in frame, expressing a fusion product of E6 and E7.

The construction of pSFV3-enh-E6E7 was done by cloning the intact E6E7 fragment and the translational enhancer-FMDV A2 autoprotease fragment in pSFV3. Since E6 and E7 are not in frame it is to be expected that this plasmid encodes E6 in an enhanced fashion, while translation of E7 is not enhanced. The inserts encoding enhE6E7 and enhE6,7 were sequenced to verify that no modifications had been generated during the PCR.

Production and purification of recombinant SFV particles

pSFV3-LacZ was obtained from Life Technologies. The pSFV3-E6E7, pSFV3-enhE6E7, pSFV3-enhE6,7, pSFV3-LacZ and the pSFV-Helper 2 plasmids were isolated using the Qiagen midi plasmid purification kit (Qiagen, Hilden, Germany) and linearized by digestion with *Spe*I (Life Technologies). RNA was synthesized from the linearized DNA by *in vitro* transcription using SP6 RNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Capping analog was obtained from Life Technologies. Fifteen μ g SFV3-E6E7 or SFV3-LacZ and 7.5 μ g SFV-Helper 2 RNA were admixed and cotransfected into 8×10^6 BHK cells in 0.8 ml GMEM by electroporation using the Biorad Gene Pulser II (two pulses of 850 V/25 μ F; BioRad, Hercules, CA, USA). After pulsing, the cells were suspended in 10 ml GMEM and cultured for 36 h at 37°C and 5% CO₂. The medium, containing the SFV-E6E7 or SFV-LacZ particles was centrifuged twice in a JA 20 rotor (Beckman, St Paul, MN, USA) at 1800 r.p.m. (ie 40 000 g at r_{max}) to remove cells and cellular debris.

The SFV particles were purified on a discontinuous sucrose density gradient (2 ml of a 15% sucrose solution (w/v) and 1 ml of a 50% sucrose solution (w/v) in TNE-buffer (50 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, pH 7.4)). Virus was collected from the interface. Sucrose was removed from the virus solution by overnight dialysis against TNE buffer. The virus suspension was concentrated approximately 10-fold (Centricon 30 filter; Millipore, Bedford, MA, USA), quickly frozen in N₂ and stored in aliquots at -80°C.

Before use, SFV particles were incubated with 1/20 volume of α -chymotrypsin (10 mg/ml; Sigma Chemical, St Louis, MO, USA) for 30 min at room temperature to cleave the mutated spike proteins. Subsequently, α -chymotrypsin was inactivated by the addition of 0.5 volume of aprotinin (2 mg/ml; Sigma Chemical).

Titer determination of SFV particles

Recombinant SFV particles were titrated by serial dilution on monolayers of BHK cells. After infection and overnight incubation, the cells were fixed for 10 min in 10% acetone and stained using a polyclonal rabbit anti-replicase (nsP3) antibody (a kind gift from Dr T Ahola, Biocentre Viiki, Helsinki, Finland) as primary antibody and FITC-labeled goat-anti-rabbit IgG as a secondary antibody (Southern Biotech, Birmingham, AL, USA). Positive cells were counted and the titer was determined after correcting for the dilution factor and the dilution caused by the activation and the volume of particles added.

Analysis of E6 and E7 expression by Western blotting

BHK cells were infected with SFV-E6E7, SFV-enhE6,7 or SFV-enhE6E7 particles. After overnight incubation, the cells were harvested and lysed in lysis buffer (50 mM Tris-Cl, 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, pH 7.4). Equal amounts of cell-free extracts were analyzed by SDS-PAGE in the presence of beta-mercaptoethanol. The proteins were blotted on to PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA). E6 and E7 were detected with a polyclonal rabbit anti-HPV16 E6 antibody (a kind gift from Dr I Jochmus, Deutsches Krebsforschungszentrum, Heidelberg, Germany) and a monoclonal mouse-anti-HPV16 E7 antibody (Zymed Lab, South San Francisco, CA, USA), respectively. After incubation with alkaline phosphatase-linked secondary antibodies, the blots were stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical).

Analysis of E6 and E7 expression by pulse-labeling

For pulse-labeling, BHK cells were infected with SFV-E6E7, SFV-enhE6E7 or SFV-enhE6,7 particles. After 6 h, the medium was removed, the plates were washed three times with phosphate-buffered saline (PBS) and the cells were cultured for an additional 30 min with methionine- and cysteine-free DMEM (ICN Biomedicals). At this time-point ³⁵S-methionine/cysteine (0.37 MBq/well; Amersham) was added to the cultures. After 1 h, the wells were washed free from radioisotope and harvested directly or cultured for an additional 6, 16 or 40 h before harvesting. At these time-points, the cells were washed with PBS (4°C), harvested by scraping and resuspended in lysis buffer containing 0.2 mM phenyl-methane-sulphonyl-fluoride. After centrifugation the supernants of the cell lysates were analyzed by SDS/PAGE and autoradiography.

Immunizations

Mice were immunized subcutaneously (s.c.) or intraperitoneally (i.p.) with 10^5 to 5×10^6 recombinant SFV particles, followed by one or two booster immunization with a 2-week interval. As negative controls, mice were injected with equal doses of SFV-LacZ particles or PBS.

CTL assay

Seven days to 3 months after the last booster immunization, spleen cells were isolated and cocultured with irradiated (100 Gy) TC-1 cells in a ratio of 25:1, in 25 cm² culture flasks, placed upright. After 1 week in culture, cells were harvested and a CTL assay was performed by a standard 4-h ⁵¹Cr release assay in triplicate determi-

nations. Target cells were labeled for 1 h with 3.7 MBq $^{51}\text{Cr}/10^6$ cells in 100 μl medium (^{51}Cr was from Amersham, London, UK). The mean percentage of specific ^{51}Cr release of triplicate wells was calculated according to the formula: % specific release = ((experimental release - spontaneous release)/(maximal release - spontaneous release)) c.p.m. \times 100. The spontaneous ^{51}Cr release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.

Initially, CTL analysis was also performed after an additional period of *in vitro* stimulation. For these experiments spleen cells were initially cultured as described above. After 1 week, the cells were harvested and restimulated with irradiated naive spleen cells (30 Gy) and irradiated C3 cells in a ratio of 2.5:0.1 in 24-well plates in the presence of 4 IU of recombinant hIL2/ml (Strathmann Biotech, Hamburg, Germany). Five days after restimulation, cells were harvested and a ^{51}Cr release assay was performed as described above.

Precursor CTL frequency determination by IFN- γ Elispot analysis

Elispot analysis was done essentially according to the method described by Miyahira et al.⁴⁷ In short, serially diluted, known numbers of freshly isolated spleen cells were plated into wells (96-well high affinity plates, Greiner) that had been coated overnight with purified anti-mouse-IFN- γ mAb (Pharmingen, San Diego, CA, USA). Subsequently, 13-2 cells (only expressing the HPV16 E7₄₉₋₅₇ CTL epitope) were added for *in vitro* restimulation using effectors to stimulator cell ratios of 1:1 to 4:1. In addition, spleen cells were cultured without stimulator cells as controls to determine antigen-independent IFN- γ secretion. After overnight incubation, the wells were washed extensively and incubated with biotinylated anti-mouse IFN- γ mAb (Pharmingen). After a 1-h incubation at 37°C, the plates were washed and streptavidin-alkaline phosphatase was added. After a 1-h incubation at 37°C, the plates were washed and the spots were developed by adding the substrate BCIP (Sigma) in agarose. After overnight incubation at 4°C, the number of spots was determined using a stereomicroscope. Spots were counted in nine wells that contained between 10 and 100 spots per well. The number of spots in three wells containing equal numbers of unstimulated cells was subtracted from the numbers counted in wells containing stimulated wells. In the experiments described in Results, the number of background spots was always less than two per 4×10^5 cells.

Tumor challenge experiments

Mice were immunized and boosted as described above with 10^6 to 5×10^6 SFV-E6E7, SFV-enhE6,7 particles, SFV-LacZ particles or PBS. One week after the last booster immunization, the mice were challenged s.c. in the neck with 2×10^4 TC-1 cells suspended in 0.2 ml Hanks buffered salt solution (Life Technologies). Tumor measurements were always done by the same skilled technician. Once tumor volume reached approximately 1000 mm³, the mice were killed.

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